The colorimetric determination of ester groups in lipid extracts

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► The determination of ester groups in lipid extracts is usually carried out by the alkaline hydroxylaminolysis of esters to form hydroxamic acids which, on treatment with a ferric ion in acid solution, yield highly colored Fe^{+++}-chelate complexes. The method of Stern and Shapiro (1), which is widely used for the routine examination of the lipids of blood serum, employs aqueous conditions for the initial reaction with hydroxylamine. This results in a low color yield in the subsequent reaction with Fe^{+++} ions, and furthermore, cholesterol liberated from its esters is insoluble in the final reaction mixture and produces cloudy solutions. These two shortcomings, which have made the method unsuitable for sera containing either very low or very high lipid concentrations, have been largely overcome by the method of Rapport and Alonzo (2), which employs anhydrous conditions. This latter method, however, has not proved suitable for the routine investigation of ester groups since each reaction tube has to be treated individually.

We have modified the method of Stern and Shapiro (1) to employ relatively nonaqueous conditions both for the initial hydroxylaminolysis, and for the subsequent color development, using alcoholic ferric perchlorate solution instead of aqueous ferric chloride. This has resulted in the production of high color yields which are stable; the method is precise, accurate, and suitable for the routine investigation of a large number of lipid extracts.

The color formation obeys Beer's law up to the range of 4 μeq as shown in Figure 1; however, the intensity

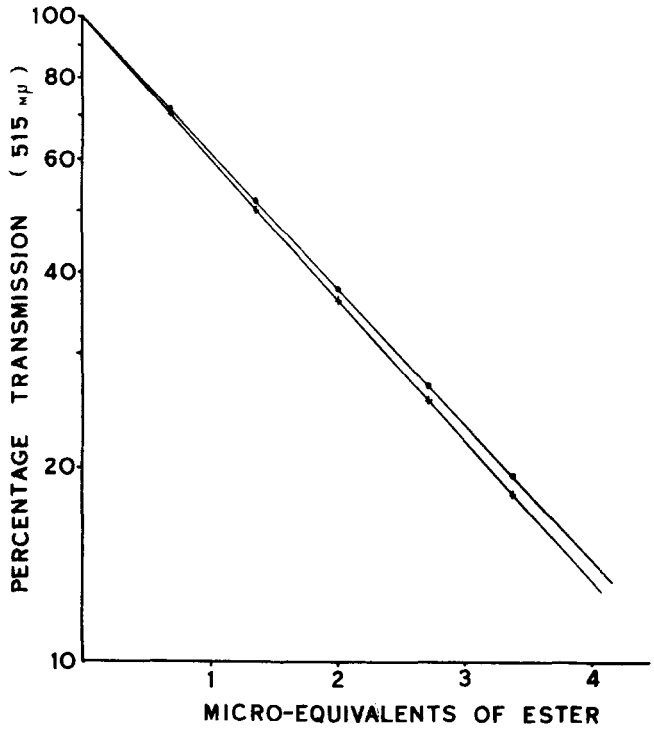

FIG. 1. The relationship between ester concentration and percentage transmission at 515 mμ. O——O = curve for cholesteryl acetate standard; X——X = curve for triolein standard.

of maximum absorption at 515 mμ is slightly lower for cholesterol esters than for triglycerides (95%).

The method has an average error of 1.7% (range 0% to 5.5%). This has been determined on duplicate aliquots of lipid extracts derived from 25 sera covering the range 5 to 20 meq of ester per liter of serum.

Peroxide-free isopropyl ether is recommended instead of diethyl ether as the solvent for the initial reaction because of its lower volatility (b.p. 67.5°).

Reagents: All reagents and solvents used are Anal. Reagent grade. Isopropyl ether is freed from peroxides by passage through a column of activated alumina (heated overnight at 170°) just before use.

Methanolic hydroxylamine solution (2 M). (A): Add 13.9 g hydroxylamine hydrochloride to 5 ml distilled water and 50 ml of absolute methanol. Warm until dissolved and make up to 100 ml with methanol.

Methanolic NaOH solution (3.5 N). (B): Add 14 gm of NaOH to 5 ml distilled water and 50 ml methanol. Warm until dissolved and make up to 100 ml with methanol.

Alkaline hydroxylamine solution: Mix equal parts by volume of (A) and (B) and allow to stand for 15 minutes. The clear supernatant is filtered off just before use.

Stock ferric perchlorate solution: Dissolve 1.0 gm of iron wire in 125 ml of 70% to 72% perchloric acid in a beaker by warming gently on a hot plate until the reaction begins, then remove from the source of heat. N.B. Care must be taken as the reaction may become very vigorous. When solution is complete, make up to 250 ml with distilled water and store at 4°. Allow the solution to stand for 3 to 4 days before use. The solution is stable for at least 2 weeks.

Working ferric perchlorate solution: Dilute 6 ml of the stock to 120 ml with absolute ethanol just before use.

Procedure. Aliquots of lipid extracts containing from 0.5 to 4.0 μeq of ester (3 ml aliquots of 1:25 serum extracts are suitable) are evaporated to dryness in Evelyn tubes on a hot-water bath with an air blower. Then 3 ml of isopropyl ether and 1 ml of the alkaline hydroxylamine solution are added, the tubes are stoppered (polythene closure), well shaken, and allowed to stand at room temperature for one-half hour. Six ml of the working ferric perchlorate solution is then added; the tubes are again stoppered, well shaken, and allowed to stand in a dark cupboard for a further half hour. A blank (3 ml isopropyl ether) and standards (containing 1 to 4 μeq of ester) are carried through the procedure.

The color densities of the solutions are read on an Evelyn colorimeter using the 515 mμ filter, the 6 ml aperture, and the bright light source with a fully polished colorimeter reflector. The instrument is initially set at 100% transmission with the blank.

The procedure described has been developed to meet the requirements of the Evelyn colorimeter, which has a light path of 2 cm and requires a minimum volume of 6 ml of solution for measurement of the color density. The final volume (10 ml) has also been adjusted so that a reading of 20% transmission (equivalent to an optical density of 0.7) is produced by 3 μeq of ester. With colorimeters or spectrophotometers having a 1 cm light path, the color density readings are halved for a given ester concentration. The range of standards may be extended considerably, however, and when determined with a Beckman D.U. Spectrophotometer having a light path of 1 cm, the graph of percentage transmission at 515 mμ against ester concentration, plotted on semilogarithm paper, is perfectly linear up to 8 μeq of ester. It is preferable when using such instruments to reduce the final volume to 5 ml by using half quantities of all the reagents specified for the original proportion of standards or aliquots of serum extracts, i.e., 1.5 ml isopropyl ether, 0.5 ml alkaline hydroxylamine solution, and 3 ml of ferric perchlorate solution. The graph of percentage transmission against ester concentration has been determined under these conditions and is identical to that shown in Figure 1.

Snyder and Stephens (3) have recently described a method similar to ours, employing relatively anhydrous conditions. The color yield produced in their method, as well as in that of Rapport and Alonzo (2), has been determined with a 1 cm light path and is almost identical to that obtained in our procedure when adjusted for a 1 cm light path as described above. These methods differ from ours in that they require heating at 65° in order to ensure quantitative hydroxylaminolysis of the ester. This is a disadvantage, however, since the reaction is very sensitive to the temperature and duration of the heating period, and, as a result, these methods lack the precision and accuracy obtained when the reaction is allowed to proceed at room temperature.

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